

Efficiency of Human Rotavirus  
Propagation in Cell Culture

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| 16. ABSTRACT<br><p>This study was designed to find methods to reproducibly propagate human rotaviruses from fecal specimens and to determine the relationship between particle numbers and infectivity. Growth of virus was initially compared in primary and continuous lines of monkey kidney cells. Primary cells (African green and cynomolgus monkey kidney) supported virus growth directly from fecal specimens much more efficiently than continuous lines of African Green (CV-1) or rhesus (MA104) monkey kidney cells. Rotaviruses were grown in primary cells from 14/14 fecal specimens of different individuals collected over a three-year period. Although rotaviruses in fecal samples could not always be grown in the continuous cell lines, two passages in primary cells appeared to fully adapt the viruses for propagation in the continuous cell line tested (MA104). The efficiency of rotavirus growth was quantified with five of the fecal isolates. It was calculated that, on the average, 1 out of every 46,000 passages in primary cells an average of 1 out of every 6,600 progeny virus particles appeared to be infectious. Thus, rotaviruses in fecal specimens were consistently grown in primary cells and passage in these cells both increased virus infectivity and adapted the viruses for growth in continuous cell lines.</p> |                                                  |                                          |  |
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## Efficiency of Human Rotavirus Propagation in Cell Culture

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This study was designed to find methods to reproducibly propagate human rotaviruses from fecal specimens and to determine the relationship between particle numbers and infectivity. Growth of virus was initially compared in primary and continuous lines of monkey kidney cells. Primary cells (African green and cynomolgus monkey kidney) supported virus growth directly from fecal specimens much more efficiently than did continuous lines of African green (CV-1) or rhesus (MA104) monkey kidney cells. Rotaviruses were grown in primary cells from 14 of 14 fecal specimens of different individuals collected over a 3-year period. Although rotaviruses in fecal samples could not always be grown in the continuous cell lines, two passages in primary cells appeared to fully adapt the viruses for propagation in the continuous cell line tested (MA104). The efficiency of rotavirus growth was quantified with five of the fecal isolates. It was calculated that, on the average, 1 out of every 46,000 particles in fecal specimens infected monkey kidney cells. After three passages in primary cells, an average of 1 out of every 6,600 progeny virus particles appeared to be infectious. Thus, rotaviruses in fecal specimens were consistently grown in primary cells, and passage in these cells both increased virus infectivity and adapted the viruses for growth in continuous cell lines.

Rotaviruses are known to be a major cause of acute diarrheal disease in the young of numerous species, including humans. Although a number of mammalian rotavirus isolates had been grown in cultured cells, no human rotavirus was successfully cultivated until quite recently. The first human isolate to be grown in cultured cells had been passaged 11 times through gnotobiotic piglets (18). This strain, called Wa, has now been grown in a number of primary cell types and continuous cell lines.

Several groups have recently demonstrated that human rotaviruses can be grown directly in monkey kidney cells without passage through animals (1, 4, 14, 16). Most investigators have reported successful cultivation of human rotaviruses in MA-104 cells, a rhesus monkey kidney cell line. Hasegawa et al. (4) have indicated that primary cynomolgus monkey kidney cells were more sensitive than MA-104 cells for propagation of human rotaviruses; and more recently Wyatt et al. (17) suggested that primary African green monkey kidney (AGMK) cells may be more efficient for cultivation of human rotaviruses from stools than MA-104 cells. All successful human rotavirus isolations in cultured cells have been achieved with culture media containing trypsin and no serum.

In an attempt to find the most efficient conditions for propagation of these viruses in cultured cells, several types of monkey kidney cells, including both continuous lines and primary cells, were compared for their abilities to support the growth of human rotaviruses from fecal specimens. Once an efficient cultivation method was found, the specific infectivity of virus particles in fecal specimens was determined. Likewise, a method was found to adapt these viruses for efficient growth in less permissive cells. Finally, relationships between the different cultured viruses were determined through electrophoretic analysis of their RNA segments.

### MATERIALS AND METHODS

**Cells.** Five types of monkey kidney cell cultures were used for these experiments. Primary cynomolgus and AGMK

cells, grown in the presence of simian virus 40 and simian virus 5 antisera, were purchased from Flow Laboratories, Inc., Rockville, Md. These were used for virus propagation between 1 and 4 days after arrival in our laboratory. Two types of MA-104 (rhesus monkey kidney) cells were used, one obtained from E. A. Bohl, Ohio Agricultural Research and Development Center, Wooster, Ohio (MA104-A), and the other from M. K. Estes, Baylor College of Medicine, Houston, Tex. (MA104-B). These differed in several properties, including morphological features, density at confluence, adhesion properties, and ability to support virus growth. The other cell line used was CV-1 (AGMK), obtained from S. L. Wechsler of the Christ Hospital Institute of Medical Research. All cells except CV-1 were grown in Eagle minimal essential medium (MEM) with 10% fetal calf serum and antibiotics (100 U of penicillin, 100 µg of streptomycin, and 2.5 µg of fungizone per ml). The CV-1 cells were grown in Special MEM (Richter's modification [Irvine Scientific Co., Santa Ana, Calif.]) with the same supplements.

**Viruses.** All but one of the fecal specimens containing rotaviruses used in this study were collected from patients at Children's Hospital, Cincinnati, Ohio, during the winters of 1982 and 1983 and stored at -70°C. The remaining fecal sample was from an adult with acute gastroenteritis who became ill during a waterborne outbreak in Vail, Colorado, in March 1981. The laboratory strains of rotavirus were kindly provided by M. K. Estes, Baylor College of Medicine (SA-11), and R. G. Wyatt, National Institutes of Health, Bethesda, Md. (Wa).

**Propagation of human rotaviruses.** Fecal specimens were processed by two separate methods before inoculation of cells. For both methods, 15% suspensions were made by blending with Earles balanced salt solution for 2 min at 4°C. For the first method, this step was simply followed by centrifugation (8,000 × g, 20 min) to remove most suspended solids and bacterial contaminants. The second method consisted of several steps. The blended material was mixed with an equal volume of Freon (1,1,2-trichloro-1,2,2-trifluoroethane) and blended an additional 2 min at 4°C, followed by centrifugation at 2,000 × g for 10 min to separate the phases. The aqueous phase was vigorously mixed with ether to

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decontaminate the samples, and the dissolved freon was removed with the separated ether phase. Excess ether was evaporated by bubbling  $N_2$  throughout the samples. Specimens were processed only by the first of these two methods during the latter phases of this study.

Processed fecal specimens were incubated at 37°C for 30 min in the presence of 15  $\mu$ g of trypsin (1:250; GIBCO Laboratories, Grand Island, N.Y.) per ml, and 0.2-ml samples were inoculated onto monkey kidney cells in culture tubes. The cells had been washed three times with Earles balanced salt solution to remove serum before inoculation. After the tubes were rolled for 2 h at 37°C to permit virus adsorption, the cells were washed twice with Earles balanced salt solution to remove fecal contaminants. MEM (2 ml) with antibiotics and 2  $\mu$ g of trypsin per ml was then added, and the culture tubes were rolled for an additional 5 days or until the cytopathic effect (CPE) was essentially complete. Tubes were then stored frozen until assayed or used to make further passages. Additional passages were performed in the same fashion except that trypsin pretreatment was not used and cells were not washed after the adsorption period. Many of these methods are similar to those used by other investigators (1, 4, 14, 16, 17) to propagate human rotaviruses from fecal specimens.

**Assay for rotavirus antigens.** The presence of rotavirus antigens was determined by an enzyme-linked immunosorbent assay (ELISA). The immunoglobulins for this assay were made by Dakopatts A/S, Copenhagen, Denmark. All procedures were described in the manual that accompanies the ELISA kit distributed by Accurate Chemicals, Westbury, N.Y. In brief, immunoglobulins from rabbits taken both before and after inoculation with human rotaviruses were adsorbed overnight at 4°C in separate wells of microtiter plates. After addition and adsorption of viruses, blocking antibodies and peroxidase-conjugated antirotavirus antibodies were added. After a 15-min incubation with substrate (*o*-phenylenediamine), the reaction was stopped with  $H_2SO_4$  and the colorimetric reaction was measured by absorbance at 490 nm with an automated reader (Litton Bionetics, Inc., Charleston, S. C.). The absorbance values of control wells were normally about 0.06, and positive values were always at least twice those of the controls.

**Electron microscopy.** Determination of rotavirus concentrations through particle count measurements was performed by Betty Petrie, Baylor College of Medicine. Samples to be analyzed were transported on dry ice, and particle numbers were determined by the pseudoreplication technique (8). More than 90% of the particles in each preparation contained the double protein shells required for infectivity (2), except unpassaged fecal sample 9, which contained about 80% particles with double protein shells.

**Fluorescent focus assay.** This assay for infectious virus was performed with MA104-B cells on round cover slips (22-mm diameter; Carolina Biologicals, Inc., Burlington, N.C.). Cover slips were washed in ethanol, heat sterilized, and placed in individual wells of 12-well cluster dishes, to which cells were added and grown to confluency. Rotaviruses in fecal specimens were incubated with 20  $\mu$ g of trypsin per ml for 30 min at 37°C and then diluted at least 10-fold in Special MEM with antibiotics and 2  $\mu$ g of trypsin per ml before inoculation of cells. Viruses passaged through cells were not pretreated with trypsin before dilution. After inoculation with 0.5 ml of sample per well, incubation was continued for 24 h at 37°C. Cover slips were then immersed in acetone at -20°C for 10 min and dried. Infectious centers were measured by the indirect fluorescence method. For this, 0.1 ml

of guinea pig antirotavirus hyperimmune serum (1:400 dilution) was incubated on each cover slip at 37°C for 30 min and washed by immersion in phosphate-buffered saline. Fluorescein-conjugated goat anti-guinea pig immunoglobulin G (1:80 dilution; Cappel Laboratories, Dowingtown, Pa.) was added (0.1 ml), and cover slips were incubated for 30 min at 37°C before again being washed with phosphate-buffered saline. These were then mounted on slides with 90% glycerol in phosphate-buffered saline, and infectious centers (foci) were counted. Sufficient fields were viewed to account for 2 to 9% of the area of each cover slip, and the number of foci per milliliter of original sample was calculated. The limit of detection by this technique was about  $5 \times 10^2$  infectious units per ml of sample.

**Labeling, extraction, and polyacrylamide gel analysis of rotavirus RNA.** Gel analysis of rotavirus genome segments was performed on RNA extracted from [ $^3H$ ]uridine-labeled virus preparations. All 14 rotavirus isolates from fecal specimens, as well as two laboratory strains (SA-11 and Wa), were analyzed. The 14 isolates were obtained after several passages in primary AGMK cells, followed by additional passages in MA104-B cells. Fecal isolates 1 through 4 were passed eight times in primary cells and nine times in MA104-B cells, and isolates 5 through 14 were passed three times in primary cells and four times in MA104-B cells. These, along with the SA-11 and Wa rotavirus strains, were used to infect confluent monolayers of MA104-B cells in 150-cm<sup>2</sup> flasks washed three times with Earles balanced salt solution before inoculation (3.5 ml of virus per flask). After the 2-h adsorption period at 37°C, 15 ml of MEM with antibiotics and 2  $\mu$ g of trypsin per ml was added, and incubation was continued. [ $^3H$ ]uridine (0.2 mCi) was added to each flask after both 4 and 28 h of incubation. The flasks were then incubated and rocked a total of 3 days after infection. Significant CPE was observed in most samples. The flasks infected with SA-11 and Wa viruses were harvested within 2 days after infection when CPE was complete. The flasks were frozen and thawed to dislodge cells, and the contents of the flasks were centrifuged (120,000  $\times$  g, 90 min) to pellet viruses and debris. The nucleic acids were extracted with phenol, precipitated from 70% ethanol by centrifugation (1,500  $\times$  g, 20 min), and suspended in STE buffer (0.1 M NaCl, 0.01 M Tris, pH 7.2, 0.001 M EDTA), all according to methods described by Pons (9, 10). Ethanol was added (final concentration: 30% [vol/vol]), and the double-stranded RNAs were separated from other nucleic acids by chromatography on CF-11 (Whatman cellulose powder) columns (9, 10). After precipitation from ethanol, the samples were dissolved in electrophoresis sample buffer (0.01 M NaCl, 0.005 M Tris, pH 7.4, 0.005 EDTA, 0.05% sodium dodecyl sulfate, 15% sucrose) and electrophoresed in polyacrylamide slab gels (1-mm thickness) according to the methods of Laemmli (5), except that the electrode buffer was 0.05 M Tris-0.38 M glycine-0.1% sodium dodecyl sulfate. By using a 4% stacking gel and a 10% separation gel, electrophoresis was performed for 18 h at a constant current of 20 mA. The position of the RNA bands were determined by fluorography (6) after impregnation of the gels with 2,5-diphenyloxazole.

## RESULTS

**Comparative efficiencies of different monkey kidney cells in propagation of human rotaviruses from fecal specimens.** Monkey kidney cells from five different sources were used to propagate human rotaviruses from fecal specimens. Rotaviruses in the four specimens examined in the initial study were extracted by both a blending-centrifugation and a

TABLE 1. Presence of rotavirus antigens from fecal specimens after 10 passages in monkey kidney cells as determined by the ELISA

| Fecal sample | Extraction method <sup>a</sup> | Cell line and dilution <sup>b</sup> |   |   |         |   |   |      |   |   |      |   |   |
|--------------|--------------------------------|-------------------------------------|---|---|---------|---|---|------|---|---|------|---|---|
|              |                                | MA104-A                             |   |   | MA104-B |   |   | CV-1 |   |   | AGMK |   |   |
|              |                                | 0                                   | 1 | 2 | 0       | 1 | 2 | 0    | 1 | 2 | 0    | 1 | 2 |
| 1            | B-C                            | -                                   | - | + | +       | + | + | +    | + | + | +    | + | + |
|              | B-F-E                          | +                                   | - | - | +       | - | + | +    | + | - | +    | + | + |
| 2            | B-C                            | -                                   | - | - | -       | - | - | -    | - | - | -    | + | - |
|              | B-F-E                          | -                                   | - | - | -       | - | - | -    | - | - | +    | + | + |
| 3            | B-C                            | -                                   | + | - | +       | - | - | -    | + | - | +    | + | + |
|              | B-F-E                          | -                                   | - | - | -       | - | - | +    | + | + | +    | + | + |
| 4            | B-C                            | -                                   | - | - | -       | - | - | +    | - | - | +    | + | + |
|              | B-F-E                          | -                                   | - | + | -       | - | - | +    | + | + | +    | + | + |

<sup>a</sup> B-C, Blending-centrifugation; B-F-E, blending-Freon-ether.<sup>b</sup> The log<sub>10</sub> dilution of the original stool preparation.

blending-Freon-ether procedure as described above, treated with trypsin, and either inoculated directly onto washed cells in culture tubes or diluted one or two orders of magnitude (logs) before inoculation. Thus, each specimen was processed by two methods and inoculated at three separate dilutions onto each of five monkey kidney cells types, a total of 120 culture tubes.

All inoculated cultures were examined for toxicity immediately after the 2-h adsorption period. Several of the undiluted virus preparations processed by the blending-centrifugation procedure caused almost complete cell destruction by this time. Cell destruction by samples treated by the blending-Freon-ether procedure was less obvious, and primary cells were less affected than the continuous cell lines. None of the culture tubes inoculated with diluted specimens displayed cytotoxicity at the end of the adsorption period.

Each of the 120 inoculated cultures was passaged 10 times and then tested by the ELISA to detect rotavirus antigens. A number of the passaged cultures from each of the continuous cell lines were positive, but many were negative (Table 1). Rotavirus antigens were not detected in specimen 2. In contrast, almost all of the undiluted, as well as diluted, specimens passaged in primary cells were strongly positive for rotavirus. Thus, primary cells supported rotavirus growth from these four stool samples better than the continuous cell lines. It should be noted, however, that obvious CPE was found only in the continuous cells and cultures that displayed consistent CPE for several passages were all positive for rotavirus at the tenth passage.

**Adaptation of human rotavirus for growth in continuous cell lines by passage in primary cells.** Because growth of human rotaviruses was less consistent in the continuous cell lines than in primary monkey kidney cells, the possibility that passage through primary cells may adapt these viruses for consistent growth in continuous cell lines was examined. In an initial experiment, viruses from fecal specimens 1 through 4 were inoculated onto the three continuous monkey kidney cell lines after eight passages in primary AGMK cells. After 2 additional passages in the continuous lines, all samples that were positive for rotavirus by the ELISA after 10 passages in the AGMK cells were also strongly positive in all three cell lines (absorbance at 490 nm > 1.0). This result must reflect virus growth because comparable dilutions of the inocula (eighth-passage AGMK cultures) were only weakly positive (absorbance at 490 nm < 0.3). In addition, significant CPE was found in many of the MA104-B cultures during both passages. Thus, passage of human rotaviruses in primary AGMK cells appeared to adapt them for consistent growth in continuous cell lines.

To determine the number of passages in primary monkey kidney cells required for adaptation of human rotavirus from fecal specimens for growth in a continuous line of monkey kidney cells, the original fecal preparations, numbered 1 through 4, were again inoculated onto primary AGMK cells. This time, however, cultures from the first passage were used to inoculate both primary AGMK cells and MA104-B cells. MA104-B cells were selected because they had displayed greater CPE than the other two continuous cell lines

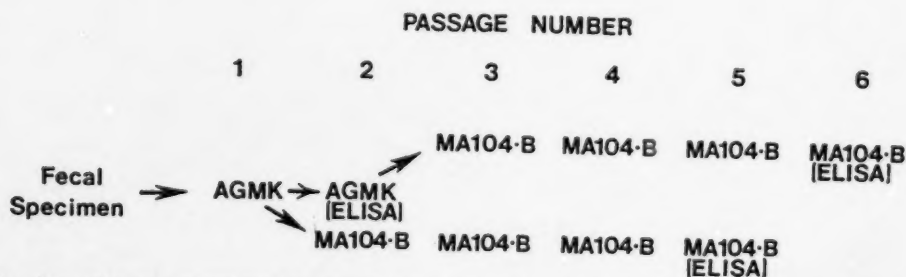


FIG. 1. Outline of experimental protocol used to determine the number of passages in primary AGMK cells required to adapt human rotaviruses in fecal specimens for growth in MA104-B cells.

TABLE 2. Adaptation of human rotaviruses from fecal specimens for growth in MA104-B cells after one or two passages in primary AGMK cells as determined by the ELISA

| Fecal sample | Extraction method <sup>a</sup> | Passage history (cell line and dilution) <sup>b</sup> |   |   |                                              |   |   |                                               |   |   |
|--------------|--------------------------------|-------------------------------------------------------|---|---|----------------------------------------------|---|---|-----------------------------------------------|---|---|
|              |                                | AGMK (two passages)                                   |   |   | AGMK (one passage) → MA104-B (four passages) |   |   | AGMK (two passages) → MA104-B (four passages) |   |   |
|              |                                | 0                                                     | 1 | 2 | 0                                            | 1 | 2 | 0                                             | 1 | 2 |
| 1            | B-C                            | +                                                     | + | + | +                                            | + | + | +                                             | + | + |
|              | B-F-E                          | +                                                     | + | + | +                                            | + | + | +                                             | + | + |
| 2            | B-C                            | -                                                     | - | - | -                                            | - | - | -                                             | - | - |
|              | B-F-E                          | +                                                     | - | + | -                                            | - | - | +                                             | - | + |
| 3            | B-C                            | +                                                     | + | + | +                                            | + | - | +                                             | + | + |
|              | B-F-E                          | +                                                     | + | + | +                                            | + | + | +                                             | + | + |
| 4            | B-C                            | +                                                     | + | + | +                                            | + | - | +                                             | + | + |
|              | B-F-E                          | +                                                     | + | + | -                                            | + | - | +                                             | + | + |

<sup>a</sup> B-C, Blending-centrifugation; B-F-E, blending-Freon-ether.<sup>b</sup> The log<sub>10</sub> dilution of the original stool preparation.

in the previous studies. The MA104-B cultures were passed an additional three times in these cells before analysis for rotavirus antigens by the ELISA. Second-passage AGMK cultures were also analyzed by the ELISA. In addition, these cultures were used to inoculate MA104-B cells. After three additional passages in MA104-B cells, these too were analyzed for rotavirus antigens. An outline of the passage history just described is summarized in Fig. 1.

The results of this experiment (Table 2) show that one passage through primary AGMK cells partially adapted these human rotaviruses for growth in MA104-B cells, and two passages in primary cells appeared to fully adapt these viruses for growth in the continuous cell line. That is, the same cultures were positive after an additional four passages in MA104-B cells as were positive after two passages in primary cells. Without significant virus growth in MA104-B cells, all cultures would have been ELISA negative after four passages in these cells. Instead, the amount of rotavirus antigen detected in these samples was comparable with that present after the two passages in primary cells. These results were confirmed on 10 additional fecal specimens (results not shown).

Specimens in these studies were processed by two methods, both of which permitted virus propagation. The blending-centrifugation procedure required fewer steps, but several of the undiluted samples obtained by this procedure had significant bacterial contamination. In addition, cell toxicity was greater with samples treated by this procedure. However, other experiments performed in this laboratory have

shown that Freon treatment, as was used in the second extraction procedure, can cause large reductions in the infectivity of rotaviruses (results not shown). This was especially evident when the human rotavirus strain Wa was purified with Freon. To avoid possible virucidal effects of Freon, all further fecal specimens were processed by the blending-centrifugation procedure. The use of a 1-log dilution of samples processed in this manner was found to eliminate detectable cytotoxicity and bacterial contamination problems.

**Relationship between number of viral particles and infectious rotaviruses in fecal specimens.** Five fecal specimens obtained from infected children during the winters of 1982 and 1983 were processed and examined to determine the fraction of virus particles in fecal specimens that were infectious. The processed specimens were serially diluted from 2 to 7 log<sub>10</sub> and were used to infect primary AGMK cells. After each of the first three passages, cultures were analyzed for rotavirus antigens by the ELISA. In addition, rotavirus concentrations in the five processed samples were directly measured by electron microscopy and fluorescent focus formation.

All five fecal preparations could be diluted at least five logs before inoculation of primary AGMK cells and still produce sufficient viral antigen after two passages to be easily detected by the ELISA (Table 3). Although the detection level for all five samples after only one passage was 1 log less than was found after two passages, the detection level did not change in any of the samples between

TABLE 3. Presence of human rotavirus antigens in each of the first three passages (primary AGMK cells) of fecal preparations as a function of the original log<sub>10</sub> dilution, as determined by the ELISA

| Fecal sample | Passage no. and dilution <sup>a</sup> |   |   |   |   |   |  |     |   |   |   |   |   |  |
|--------------|---------------------------------------|---|---|---|---|---|--|-----|---|---|---|---|---|--|
|              | One                                   |   |   |   |   |   |  | Two |   |   |   |   |   |  |
|              | 2                                     | 3 | 4 | 5 | 6 | 7 |  | 2   | 3 | 4 | 5 | 6 | 7 |  |
| 5            | +                                     | + | + | + | - | - |  | +   | + | + | + | + | - |  |
| 6            | +                                     | + | + | - | - | - |  | +   | + | + | + | - | + |  |
| 7            | +                                     | + | + | - | - | - |  | +   | + | + | + | - | + |  |
| 8            | +                                     | + | + | - | - | - |  | +   | + | + | + | - | + |  |
| 9            | +                                     | + | + | + | - | - |  | +   | + | + | + | + | - |  |

<sup>a</sup> The log<sub>10</sub> dilution of the original stool preparation.

passage 2 and passage 3. When the results obtained in this experiment were combined, the average concentration of cultivatable rotaviruses in these fecal preparations was found to be  $3 \times 10^6$  per ml, as determined by the method of Reed and Muench (11).

Concentrations of rotaviruses in these fecal preparations determined by fluorescent focus formation and electron microscopy are shown in Table 4. The average concentration of fluorescent focus units (FFU) ( $1.9 \times 10^6$ /ml) was very similar to that found through propagation. When the fluorescent focus results were compared with particle count data, an average of 1 out of every 46,000 particles was found to be infectious.

The extent of rotavirus growth after passage in primary AGMK cells was also determined. For this, cultures were examined that had been passaged three times after a 4-log dilution of the original inoculum. The average number of particles per milliliter in these cultures was  $2.0 \times 10^8$  (Table 5), 360-fold less than the average measured in the original fecal preparations (see Table 4). The infectivity of passaged viruses was somewhat greater than that of viruses in the original inocula, as determined by fluorescent focus formation. Thus, the average number of particles per FFU decreased from 46,000 to 6,600. This is still considerably larger than the particle per PFU ratio normally found for viruses that are more easily grown in cultured cells.

**Analysis of labeled RNA from human rotavirus isolates.** Although the human rotaviruses propagated in the studies described above were from fecal specimens of different individuals, all were obtained from Children's Hospital, Cincinnati, Ohio, and therefore were from children living within a limited geographic region. Also, these isolations were made only during the winters of 1982 and 1983. However, several groups have reported that a number of distinct rotaviruses, as determined by electrophoretic analysis of their RNA genome segments, can circulate in a community at any one time (see reference 1 for review). To determine whether only one strain or multiple rotavirus strains were cultured during this study, their RNA patterns were compared after gel electrophoresis.

The nine isolates described above were characterized, along with five others. One isolate was obtained from a fecal sample of an adult infected during a 1981 waterborne outbreak of gastroenteritis in Vail, Colorado. Each isolate was initially grown in primary AGMK cells (three or more passages), followed by passage in MA104-B cells. These viruses were then grown in MA104-B cells in the presence of [ $^3$ H]uridine, and the labeled genome segments were extracted and analyzed by gel electrophoresis. A schematic diagram of the RNA electrophoretic patterns of all 14 isolates is

TABLE 4. Relationship between particle numbers determined by electron microscopy and FFU in fecal preparations containing human rotaviruses

| Fecal sample | Particles per ml <sup>a</sup> | FFU per ml <sup>b</sup> | Particles per FFU <sup>c</sup> |
|--------------|-------------------------------|-------------------------|--------------------------------|
| 5            | $5.9 \times 10^{10}$          | $1.2 \times 10^6$       | $4.9 \times 10^4$              |
| 6            | $9.6 \times 10^{10}$          | $4.1 \times 10^6$       | $2.3 \times 10^4$              |
| 7            | $9.8 \times 10^{10}$          | $2.1 \times 10^6$       | $4.7 \times 10^4$              |
| 8            | $2.7 \times 10^{10}$          | $4.4 \times 10^5$       | $6.1 \times 10^4$              |
| 9            | $7.8 \times 10^{10}$          | $1.5 \times 10^6$       | $5.2 \times 10^4$              |

<sup>a</sup> Average,  $7.2 \times 10^{10}$ .

<sup>b</sup> Average,  $1.9 \times 10^6$ .

<sup>c</sup> Average,  $4.6 \times 10^4$ .

TABLE 5. Relationship between particle numbers determined by electron microscopy and FFU in fecal specimens diluted 4 logs and passed three times in primary AGMK cells

| Fecal sample | Particles per ml <sup>a</sup> | FFU per ml <sup>b</sup> | Particles per FFU <sup>c</sup> |
|--------------|-------------------------------|-------------------------|--------------------------------|
| 5            | $2.6 \times 10^8$             | $2.8 \times 10^4$       | $9.3 \times 10^3$              |
| 6            | $1.0 \times 10^8$             | $4.8 \times 10^4$       | $2.1 \times 10^3$              |
| 7            | $2.9 \times 10^8$             | $3.5 \times 10^4$       | $8.3 \times 10^3$              |
| 8            | $7.8 \times 10^7$             | $8.4 \times 10^3$       | $9.3 \times 10^3$              |
| 9            | $2.9 \times 10^8$             | $6.9 \times 10^4$       | $4.2 \times 10^3$              |

<sup>a</sup> Average,  $2.0 \times 10^8$ .

<sup>b</sup> Average,  $3.8 \times 10^4$ .

<sup>c</sup> Average,  $6.6 \times 10^3$ .

shown in Fig. 2. The RNA profiles of laboratory strains SA-11 and Wa are included for comparison. Rotaviruses numbered 1 through 7 and 10 were obtained from Children's Hospital in the winter of 1982 and those numbered 8, 9, and 11 through 13 were collected at the same location in the winter of 1983. Isolate 14 was from the individual in Colorado.

Distinct differences were found between most profiles, especially in RNA segments 5 through 9. No significant differences were detectable between isolates 1, 2, and 5 or between isolates 7 and 12. The electrophoretic pattern of isolate 11 contains at least 12 distinct segments. This has been observed by other investigators (7, 13, 15) and may be indicative of coinfection by two or more strains of rotavirus, genetic reassortment, or mutation (3). In total, at least 11 distinct RNA profiles could be discerned from 14 isolates. All displayed the "long" pattern (12) associated with subgroup 2 strains of human rotavirus.

## DISCUSSION

Previous studies conducted in other laboratories have shown that human rotaviruses in fecal specimens could be grown in monkey kidney cells. Both continuous lines and primary cells had been used, but greater success rates were reported with the latter (4, 17). These authors reported, however, that no rotaviruses could be grown from certain positive fecal specimens, even in primary cells. The study presented here was designed to find conditions under which rotaviruses could be consistently propagated from fecal samples and to determine the proportion of virus particles in fecal material that were able to grow in cultured cells.

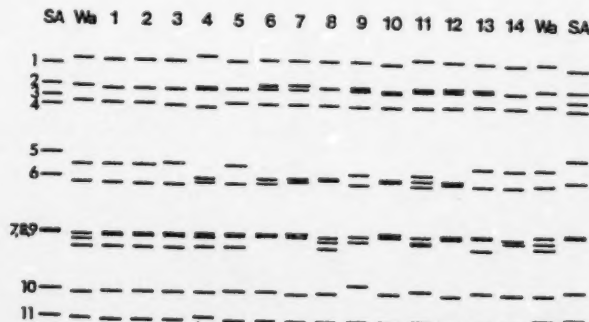


FIG. 2. Schematic diagram of the RNA segments of 14 human rotavirus isolates and strains SA-11 and Wa after electrophoresis in polyacrylamide slab gels.

Rotaviruses were propagated more efficiently in primary cells, both African green and cynomolgus monkey kidney, than in continuous monkey kidney cell line (CV-1 or MA-104) when tested at the same time and under the same conditions. This comparative study was conducted with roller tubes. For purposes of comparison, an attempt was made to propagate rotaviruses from these same fecal specimens in the continuous cell lines by using 25-cm<sup>2</sup> flasks and a rocking apparatus in place of the roller tubes. In this case, no rotavirus-positive samples were detected by the ELISA during the course of 12 passages (results not shown). Because viruses were grown from three out of four of these specimens in all three continuous cell lines (see Table 1) in roller tubes, it was concluded that the use of roller tubes is the method of choice.

Although growth of human rotaviruses directly from fecal specimens was not consistently observed in any of the continuous cell lines, these viruses could be adapted for consistent growth in these cells by passage through primary AGMK cells. A single passage through the primary cells partially adapted the viruses for growth in MA104 cells, whereas two passages appeared to allow full adaptation. It was also found that although rotaviruses in fecal specimens were propagated more efficiently in primary cells than in continuous lines, the average infectious titer of these specimens, as determined by fluorescent foci formation in MA104 cells, was nearly identical to the infectious titer determined by viral growth in primary AGMK cells. This implied that equivalent numbers of viruses in fecal specimens are able to infect the primary cells and continuous cell lines but that, in certain instances, infection of the cell lines was abortive and did not lead to the production of infectious progeny viruses. Passage through primary cells appeared to somehow overcome this abortive response. The mechanism of adaptation is not known. Possibly, either inhibitors are present in the fecal material which limit rotavirus growth in the continuous cell lines or the progeny viruses grown in primary cells are somehow different from virus particles in the stool preparations.

Passage through primary AGMK cells also increased the average infectivity of virus particles in MA104 cells, as determined by fluorescent focus formation. An average of 1 out of every 46,000 particles in fecal specimens was infectious, whereas 1 in 6,600 was infectious after three passages in primary cells.

The limit of detection of rotaviruses by the ELISA used in these studies was determined relative to particle numbers for both the processed fecal preparations and the passaged cultures. All five fecal samples tested were weakly ELISA positive after a 3-log dilution and were negative after a 4-log dilution (results not shown). Equivalent colorimetric reactions were obtained with 1-log dilutions of passaged cultures and 3-log dilutions of the original fecal preparations. Because the average particle numbers were  $7.2 \times 10^{10}$  and  $2.0 \times 10^8$ /ml in the original and passaged samples, respectively (see Tables 4 and 5), the limits of detection by the ELISA were about  $7 \times 10^7$  and  $2 \times 10^7$  particles per ml in these preparations.

The finding that about 1 particle in every 50,000 rotaviruses in fecal specimens could be grown in cultured cells and detected after two passages by an ELISA makes this a rather sensitive method for detection of rotaviruses. Other meth-

ods, including the direct ELISA and fluorescent foci assays used here, typically require greater numbers of rotavirus particles for detection. Quite possibly, this procedure can be modified so that a larger fraction of the rotavirus particles will replicate in cell culture, perhaps even as many as occur with more easily cultivatable enteric viruses such as polioviruses and reoviruses.

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